

Evaluation of the Broth Microdilution Method Using 2,3-Diphenyl-5-thienyl-(2)-tetrazolium Chloride for Rapidly Growing Mycobacteria Susceptibility Testing

As the incidence of nontuberculous mycobacterial infection has been increasing recently in Korea, the importance of drug susceptibility test for clinical isolates of mycobacteria has become larger. In this study we determined the antimicrobial susceptibility patterns of clinical isolates of *M. fortuitum* and *M. abscessus* in Korea, and evaluated the efficacy of a modified broth microdilution method using 2,3-diphenyl-5-thienyl-(2)-tetrazolium chloride (STC), in terms of its ability to provide accurate and easy-to-read minimal inhibitory concentration (MIC) endpoints for the susceptibility testing of rapidly growing mycobacteria. Most isolates of *M. fortuitum* and *M. abscessus* in Korea are susceptible or intermediately susceptible to amikacin, cefoxitin, ciprofloxacin, and clarithromycin. Many isolates of *M. fortuitum* are susceptible to doxycycline, sulfamethoxazole, and imipenem, while many *M. abscessus* isolates are resistant to these drugs. In the present study, the modified broth microdilution method using STC was found to be reliable, easy to read, and inexpensive for *M. fortuitum* and *M. abscessus* susceptibility testing. The modified colorimetric MIC testing method using STC was proven to be a useful surrogate for RGM antibiotic susceptibility testing.

Key Words : *Mycobacteria*, *Atypical*; *Mycobacterium Fortuitum*; *Mycobacterium Abscessus*; *Microbial Sensitivity Tests*; *Colorimetry*

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INTRODUCTION

Nontuberculous mycobacteria (NTM) are important environmental pathogens that cause a broad spectrum of diseases (1). NTM infections have been reported worldwide with varying frequencies (2-4), while the isolation rates and the incidences of diseases caused by NTM are increasing in Korea (5-8). In a nationwide survey from 1981 to 1994, the incidences of *Mycobacterium avium-intracellulare* complex (MAC), *M. fortuitum*, and *M. chelonae* were 65.2%, 12.7%, and 9.5%, respectively, among all NTM infections (5). And also, another report confirmed that MAC isolates were the most frequent in clinical specimens in mid-1990s, followed by *M. abscessus* and *M. fortuitum* (8). Rapidly growing mycobacteria (RGM) such as *M. fortuitum*, *M. abscessus*, and *M. chelonae* cause several forms of clinical diseases of varying severity, most commonly skin and soft tissue infections, but they also cause skeletal, pulmonary, and disseminated diseases (4, 9). In particu-

lar, MAC, *M. fortuitum*, and *M. abscessus* have been reported to be associated with clinical pulmonary diseases (6, 10-12).

NTM cause infections more commonly in the presence of predisposing factors and/or underlying diseases (4, 13) and are often considered contaminants. Because these organisms are notably resistant to the antimicrobial agents commonly used to treat tuberculosis (4, 14), susceptibility testing is indicated for any RGM that is considered clinically significant (e.g., isolates from blood, tissue, skin, and soft tissue lesions) (14, 15). The statement on the diagnosis and treatment of diseases caused by NTM issued by the American Thoracic Society suggests that a minimum of seven drugs (amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline, imipenem, and a sulfonamide) should be tested against clinically significant RGM (14), and the Clinical and Laboratory Standards Institute (CLSI) recommends that the standard broth microdilution method be used for the susceptibility testing of *M. fortuitum*, *M. chelonae*, and *M. abscessus* (15).

Although the broth microdilution procedure is widely used for the susceptibility testing of several antibiotics on a large number of bacterial isolates, several difficulties concerning MIC interpretation for RGM interfere with the susceptibility testing of these isolates (16, 17). Moreover, because the incidence of NTM infection is not high, only a few reference centers are able to offer the susceptibility testing of clinically significant strains as a service. Actually no comparative data on the susceptibility testing of these organisms is available in Korea. Thus, a more convenient and easy-to-interpret method of susceptibility testing seems important. As a modification of the CLSI standard method, the colorimetric or spectrophotometric MIC endpoint determination could be applied using color indicators. 2,3-diphenyl-5-thienyl-(2)-tetrazolium chloride (STC) (TCI, Tokyo, Japan) is an oxidation-reduction indicator that changes from colorless to red as the organisms grow, and has been applied in mycobacterial or fungal susceptibility testing (18-20). The purpose of this study was to determine the antimicrobial susceptibility patterns of clinical isolates of *M. fortuitum* and *M. abscessus* in Korea, and to evaluate the broth microdilution method using STC in terms of its ability to provide accurate and easy-to-read minimum inhibitory concentration (MIC) endpoint results for the antimycobacterial susceptibility testing of RGM.

MATERIALS AND METHODS

Antimicrobial drugs and reagents

The antimicrobial agents and their concentrations (in serial 2-fold dilutions) tested against RGM for MIC determinations were amikacin (0.5-64 $\mu\text{g}/\text{mL}$), cefoxitin (1-128 $\mu\text{g}/\text{mL}$), ciprofloxacin (0.03-4 $\mu\text{g}/\text{mL}$), clarithromycin (0.06-8 $\mu\text{g}/\text{mL}$), doxycycline (0.125-16 $\mu\text{g}/\text{mL}$), imipenem (0.25-32 $\mu\text{g}/\text{mL}$), and sulfamethoxazole (1-128 $\mu\text{g}/\text{mL}$). The drugs were purchased from Sigma Chemical (St. Louis, MO, U.S.A.), except clarithromycin (Abbott Laboratories, North Chicago, IL, U.S.A.) and imipenem (Choongwae, Seoul, Korea). Antibiotic stock solutions were made by dissolving powders in distilled water, methanol or phosphate buffer, and were divided into 1 mL aliquots and frozen at -70°C until required, except imipenem, which was used immediately after preparation. On the day of testing, antibiotic stock solutions were thawed and diluted in cation-supplemented Mueller-Hinton broth (Becton Dickinson, Sparks, MD, U.S.A.).

STC was dissolved in distilled water at 5 g/L at room temperature. This solution was filtered through a 0.20 μm filter (Corning, Corning, NY, U.S.A.) and then stored at -70°C until needed. The solubilizing agent used to dissolve reduced tetrazolium (STC) precipitates was prepared by dissolving 20% (w/v) sodium dodecyl sulfate (Bio-Rad, Hercules, CA, U.S.A.) in 50% (v/v) *N,N*-dimethylformamide (Sigma Chemical), and was stored unfiltered at room temperature.

Bacterial strains and cultures

Fifty-five *M. fortuitum* and 48 *M. abscessus* strains isolated from clinical specimens were collected from Ulsan University Hospital (UUH) and Korean Institute of Tuberculosis (KIT). Species identification was carried by high-performance liquid chromatography (strains isolated in UUH) or by polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis of the *rpoB* gene (strains provided by KIT) (14, 21-23). Strains were maintained on Löwenstein-Jensen media slants at room temperature until required for testing. To prepare the inoculum suspension for broth microdilution tests, strains were subcultured in cation-supplemented Mueller-Hinton broth and incubated at 35°C for 2 to 4 days to a turbidity of ≥ 0.5 McFarland standard by visual examination.

Staphylococcus aureus ATCC 29213 strain was tested at the beginning of each study as a quality control, as recommended by CLSI (15). Quality control was considered acceptable if MIC results were within tentative quality control ranges of MICs as recommended by CLSI (15, 16).

CLSI broth microdilution method

Antimicrobial susceptibility testing was performed by the broth microdilution method using cation-supplemented Mueller-Hinton broth, according to CLSI guidelines (CLSI broth microdilution method) (15, 17). Inoculum suspensions were prepared by diluting broth culture supernatant in sterile distilled water to a density of 0.5 McFarland standards using a nephelometer (BioMérieux Vitek Inc, Hazelwood, MO, U.S.A.). The final concentrations of mycobacteria ranged from 1×10^5 to 5×10^5 CFU/mL. Suspensions were mixed well on a vortex mixer and dispensed using a multichannel pipette into each well of a 96-well microplate (SPL, Hwasung, Korea) containing broth medium and antimicrobial drugs at serial concentrations. Final inoculums were between 1×10^4 and 5×10^4 CFU per well in a volume of 0.2 mL. Inoculated plates were covered and incubated at 35°C . A 5% sheep blood agar plate was also inoculated with a loopful of the final inoculum suspension to check for purity. If quality control results were not acceptable, or contamination or mixed culture was suspected, all data were discarded. For *M. fortuitum*, growth endpoints were read visually after 3 days of incubation. For *M. abscessus*, trays were re-incubated and read daily for up to 5 days until bacterial growth in the control well was good enough for reading. MIC values were defined as the lowest concentration of drug that inhibited visible growth, except for sulfamethoxazole, for which the concentration that inhibited growth by 80 to 90% versus a control well with no drug was used, according to the CLSI guideline (15). MIC readings for imipenem were taken at 72 hr for all strains. All measurements were performed in duplicate for each strain. To assess potential impact

on patient management, MIC results were interpreted based on the RGM interpretive category using the breakpoints suggested by CLSI.

STC broth microdilution

The colorimetric broth microdilution method with STC (STC broth microdilution) was identical to the broth microdilution method described by CLSI in terms of reagents, medium preparation, inoculum, drug concentrations, and incubation time, with one exception, namely, that STC was added to the cation-supplemented Mueller-Hinton broth with antimicrobial agents to a final concentration of 50 µg/mL. After bacterial growth, dark-colored precipitates were observed in wells containing STC-added media. After adding a solubilizing agent and incubating for 2 hr, wells showing microbial growth were pink-colored. Color changes corresponding to microbial growth and each strain-drug-MIC endpoint were recorded visually at 72 hr. The broth microdilution method using STC was checked by comparing growth in each test well with that in control wells. Wells containing the lowest drug concentration that showed a prominent reduction in color change were considered MICs. For sulfamethoxazole, wells colored light pink were considered to reflect MIC endpoints, and these showed growth inhibition of 80 to 90% versus control wells.

Comparison of the results between the CLSI and STC methods

Levels of agreement between the two broth microdilution methods with or without STC were calculated for each strain-drug-MIC endpoint and were expressed as percentages at the same MIC or as MICs within one or two 2-fold dilutions. The interpretive category results were also analyzed by comparing the results of two methods so as to determine whether the performance was acceptable according to the CLSI guideline (24). The discrepancy cases were classified to very major error (resistant by CLSI method but susceptible by STC method), major error (susceptible by CLSI method, resistant by STC method), and minor error (intermediate result was obtained by one method but not by the other). The rates of agreement were calculated as the percentage of isolates with MICs in the range of two 2-fold dilution.

RESULTS

MICs of the CLSI broth microdilution method

The MIC results determined by using the CLSI broth microdilution method are shown in Table 1 and 2 for seven drugs and a number of strains. *M. fortuitum* strains achieved maximum growth at 72 hr for the broth microdilution test meth-

Table 1. Minimal inhibitory concentrations (MICs) of 7 antimicrobial agents for 55 strains of *M. fortuitum* by CLSI broth microdilution

Agents	No. of strains distributed at the MIC (µg/mL)														
	0.016	0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256
Amikacin					51*	0	2	1	1	0	0	0	0	0	0 [†]
Cefoxitin					0*	0	0	3	8	19	20	5	0	0	0 [†]
Ciprofloxacin	29*	0	19	1	1	2	0	3	0	0 [†]					
Clarithromycin		15*	0	5	13	4	4	5	5	2	2 [†]				
Doxycycline			21*	0	1	1	5	6	9	3	3	6 [†]			
Imipenem				7*	0	12	18	13	4	1	0	0	0 [†]		
Sulfamethoxazole					4*	0	10	16	5	6	6	5	1	2 [†]	

*High off-scale MICs were converted to the next-highest concentration; [†]Low off-scale MICs were converted to the next-lowest concentration. Solid and shadowed boxes indicate susceptible and resistant categories of interpretive criteria to each antibacterial agent, respectively.

Table 2. Minimal inhibitory concentrations (MICs) of 7 antimicrobial agents for 48 strains of *M. abscessus* by CLSI broth microdilution

Agents	No. of strains distributed at the MIC (µg/mL)														
	0.016	0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256
Amikacin					5*	0	17	19	2	2	0	1	0	2 [†]	
Cefoxitin					0*	0	0	0	0	5	34	6	3	0 [†]	
Ciprofloxacin	2*	0	0	1	3	2	13	20	4	3 [†]					
Clarithromycin		19*	0	8	6	4	2	1	6	0	2 [†]				
Doxycycline			1*	0	1	2	0	5	6	3	2	28 [†]			
Imipenem				0*	0	0	1	2	1	27	12	4	1 [†]		
Sulfamethoxazole					0*	0	0	2	0	1	3	13	8	21 [†]	

*High off-scale MICs were converted to the next-highest concentration; [†]Low off-scale MICs were converted to the next-lowest concentration. Solid and shadowed boxes indicate susceptible and resistant categories of interpretive criteria to each antibacterial agent, respectively.

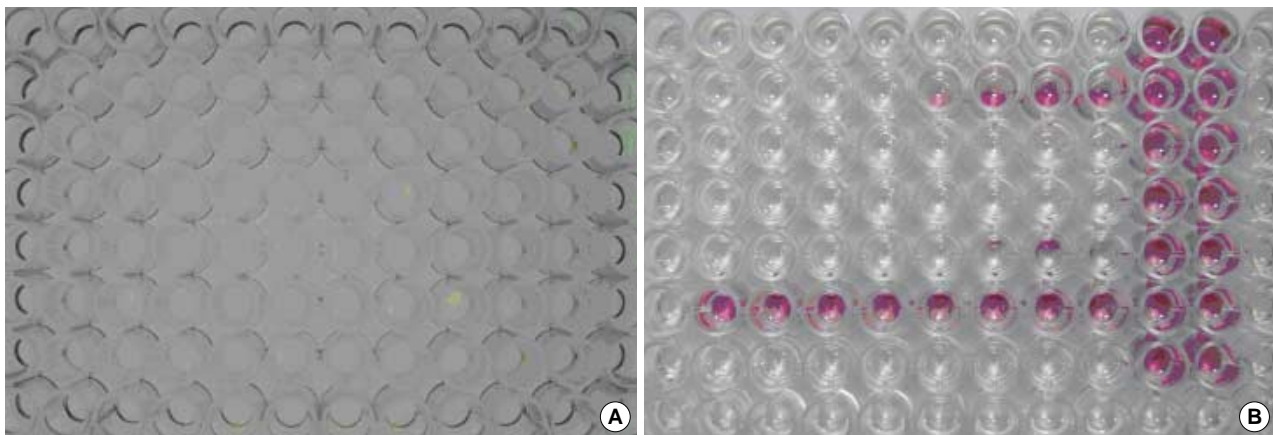


Fig. 1. Growth of *M. fortuitum* in Mueller-Hinton broth. (A) Media without STC, and (B) STC-containing media.

Table 3. Susceptibility results of rapidly growing nontuberculous mycobacteria by CLSI broth microdilution

Agents	Species (No. of strains)	<i>M. fortuitum</i> (55)			<i>M. abscessus</i> (48)		
		S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Amikacin		55 (100)	0	0	45 (94)	1 (2)	2 (4)
Cefoxitin		30 (55)	25 (45)	0	5 (11)	40 (83)	3 (6)
Ciprofloxacin		52 (95)	3 (5)	0	21 (44)	20 (42)	7 (14)
Clarithromycin		46 (84)	5 (9)	4 (7)	40 (83)	6 (13)	2 (4)
Doxycycline		28 (51)	18 (33)	9 (16)	4 (8)	14 (29)	30 (63)
Imipenem		54 (98)	1 (2)	0	4 (8)	27 (56)	17 (36)
Sulfamethoxazole		47 (85)	-	8 (15)	6 (12)	-	42 (88)

S, susceptible; I, intermediate; R, resistant.

Table 4. Agreement by MICs of antimycobacterial agents among CLSI and STC broth microdilution methods

Agents	Species	<i>M. fortuitum</i> (%)	<i>M. abscessus</i> (%)
Amikacin		54/55 (98.2)	48/48 (100)
Cefoxitin		55/55 (100)	48/48 (100)
Ciprofloxacin		55/55 (100)	47/48 (97.9)
Clarithromycin		54/55 (98.2)	46/48 (95.8)
Doxycycline		53/55 (96.4)	47/48 (97.9)
Imipenem		55/55 (100)	47/48 (97.9)
Sulfamethoxazole		48/55 (87.3)	46/48 (95.8)

MIC, minimal inhibitory concentration.

ods with or without STC. For most strains of *M. abscessus*, maximum growth in broth also occurred at 72 hr, but some strains (10 of 48) showed maximum growth at 96 hr. *M. fortuitum* showed consistently low MICs to amikacin and ciprofloxacin, and a wide range of MICs to the other five drugs. Isolates of *M. abscessus* showed a wide range of MICs to all drugs. MIC results converted to interpretive categories using the breakpoints suggested are shown in Table 3. For *M. fortuitum*, all strains were fully susceptible to amikacin, and most strains were susceptible or intermediately susceptible to cefoxitin, ciprofloxacin, clarithromycin, and imipenem. For *M. ab-*

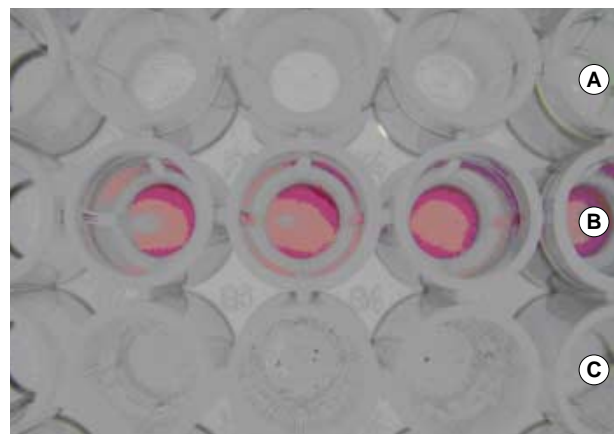


Fig. 2. Growth of *M. fortuitum* in Mueller-Hinton broth. (A) Media without STC, (B) solubilizing agent added in STC-containing media, and (C) STC-containing media, in which dark precipitates were observed before solubilization.

scnessus, most strains were susceptible or intermediately susceptible to amikacin, cefoxitin, ciprofloxacin, and clarithromycin. Doxycycline, imipenem, and sulfamethoxazole showed lower MICs for *M. fortuitum* isolates and higher MICs for *M. abscessus*.

Agreement between the CLSI and STC broth microdilution methods

Comparisons of the growth of a strain of *M. fortuitum* on the broth media are shown in Fig. 1, 2 in 96-well microplates, which demonstrate an obvious advantage of the STC broth microdilution method. The MICs of 7 antimicrobial agents obtained using the STC broth microdilution method correlated well with those obtained using the CLSI broth dilution method (Table 4). Agreements by interpretive category for the two methods are presented in Table 5. In 85 of 103 bacterial strains (44 of 55 *M. fortuitum* strains and 41 of 48 *M. abscessus* strains), the MICs of all 7 antimicrobial agents tested were within two 2-fold dilutions of those obtained by

Table 5. Agreement by interpretative category among CLSI and STC broth microdilution methods

Agents	Species (No. of strains)	<i>M. fortuitum</i> (55)			<i>M. abscessus</i> (48)		
		Minor*	Major*	Very major*	Minor*	Major*	Very major*
Amikacin		0	0	0	0	0	0
Cefoxitin		15	0	0	2	0	0
Ciprofloxacin		2	0	0	19	0	1
Clarithromycin		1	0	0	4	0	0
Doxycycline		3	0	0	5	0	0
Imipenem		1	0	0	0	1	0
Sulfamethoxazole		-	5	0	-	0	8

*Very major error, resistant by the CLSI method but susceptible by the STC method; major error, susceptible by the CLSI method but resistant by the STC method; minor error, intermediate result was obtained by one method but not by the other.

the CLSI method. For each of these agents, the overall agreement at $\pm 2 \log_2$ dilution ranged from 95.8% to 100.0% between the two methods except for the result of *M. fortuitum* to sulfamethoxazole (48 of 55 strains, 87.3%). Agreements of CLSI and STC broth microdilution methods by interpretative category results to amikacin, cefoxitin, clarithromycin, and doxycycline were excellent without major errors. Because the MIC results of *M. fortuitum* for cefoxitin were clustered at 16-32 $\mu\text{g}/\text{mL}$, the breakpoint of the susceptible and intermediate categories, 15 minor errors were identified. Similarly, the MICs of *M. abscessus* for ciprofloxacin were clustered at 1-2 $\mu\text{g}/\text{mL}$, which corresponded to 19 minor and one very major errors. In total 721 organism-antimicrobial agent combinations were analyzed, 9 of 18 MIC discrepancies (out of two 2-fold dilution), 5 of 6 major errors, and 8 of 9 very major errors originated uniquely from sulfamethoxazole test results (Table 5).

DISCUSSION

In general, the MIC results of *M. fortuitum* and *M. abscessus* isolates to antimicrobial drugs in this study were similar to those previously reported by other investigators (1, 14, 25-28). Most of the tested strains of *M. fortuitum* were found to be susceptible or intermediately susceptible to amikacin (100%), cefoxitin (100%), ciprofloxacin (100%), clarithromycin (93%), doxycycline (84%), and imipenem (100%). In addition, most strains of *M. abscessus* were susceptible or intermediately susceptible to amikacin (96%), cefoxitin (94%), and clarithromycin (96%). Ciprofloxacin and imipenem results for *M. abscessus* showed more susceptible than the results by other researchers (1, 14, 26).

It is known that results for RGM susceptibility to imipenem show a lack of reproducibility, so in a routine laboratory setting, it is recommended to repeat the test if the MIC for *M. fortuitum* is $>8 \mu\text{g}/\text{mL}$, and to report the MIC result with

the comment 'MIC greater than expected for this species' if the repeat result is $>8 \mu\text{g}/\text{mL}$ (15). In this study, no strain was found to have an MIC higher than 8 $\mu\text{g}/\text{mL}$. The MIC results of *M. abscessus* to imipenem were mainly clustered in the range 8-16 $\mu\text{g}/\text{mL}$, but the CLSI guideline suggests that MIC results should not be reported because imipenem MICs for this species tend not to be reproducible, which results in major susceptibility category changes (15, 28).

Susceptibility testing is indicated for any RGM that is considered clinically significant (e.g., isolates from blood, tissue, skin, and soft tissue lesions) (14, 15), and clinical laboratories require standardized methods for determining the antimicrobial susceptibilities of RGM. Broth microdilution is convenient and widely used for the rapid susceptibility testing to several antibiotics of large numbers of bacterial isolates. However, MICs as determined by the broth microdilution are obtained by taking visual turbidity readings, which makes the test subjective and variable (15, 17). Moreover, the growth of RGM in microtiter trays frequently does not result in a well-defined button in the bottom of a well, as occurs with rapidly growing aerobic and facultative bacteria. In addition, when testing clarithromycin susceptibility in broth, some rapidly growing mycobacteria, particularly *M. fortuitum*, had trailing end points with certain drugs.

Because of variabilities in the appearances of the growths of *M. fortuitum* and *M. abscessus* in microdilution trays, interpretation of MICs may be difficult. To avoid these drawbacks, the colorimetric redox indicator STC was used in the broth microdilution method in the present study. Results could be read visually or by spectrophotometry, and the use of a tetrazolium compound as an indicator made reading straightforward because of the obvious color change (19, 20), which overcomes the problems frequently encountered in the conventional broth microdilution test, such as, inoculum sedimentation or scant or transparent growth (20, 29). Several attempts have been made to use colorimetric determinations of broth microdilution endpoints. These methods are attractive in that they generate clear-cut endpoints based on visual color changes. STC is an oxidation-reduction indicator which, in the presence of growing organisms, changes from colorless to pink. Furthermore, it has been used to test the susceptibilities of *M. tuberculosis* to antimycobacterial drugs (18, 19). Moreover, it has been shown to have some benefits when compared with other colorimetric redox indicators, such as 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) or 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino) carbonyl]-2H-tetrazolium hydroxide (XTT) (30, 31). However, the use of these tetrazolium derivatives has several drawbacks. In MTT assays, a large number of cells are necessary (32), and XTT is expensive for routine use in clinical laboratories. Additionally for MTT and XTT methods, substrate should be added after growth has begun, and the time of this addition is not well defined (33), and in our experience the developed color disappears within

a day. In the modified susceptibility testing method using STC, the substrate can be added to media at the time of preparing media before inoculating bacteria. The plate agitation process can be eliminated, which is necessary in the MTT and XTT methods and may cause reading errors. In the present study, an excellent correlation was obtained between the results of the CLSI and STC methods. Especially the MIC and interpretive category results obtained from sulfamethoxazole susceptibility tests showed some discrepancies. We consider that the lack of an intermediate category of interpretation criteria causes major or very major errors even for a 2-fold dilution MIC difference. The CLSI guideline recommends that MIC results of sulfamethoxazole should be read at the concentration in which there is 80% or greater reduction in growth as compared to the control because antagonists in the medium may allow some slight growth (15, 28). Actually small growth of organism in microwell could be skipped in the CLSI broth microdilution method while more easily detected by color change of media in the STC method. Thus sulfamethoxazole susceptibility results should be cautiously interpreted in both broth microdilution methods.

In this study, we describe the general susceptibility patterns of *M. fortuitum* and *M. abscessus* in Korea. In addition, it was demonstrated that the STC broth microdilution method is reliable, easy to perform, and inexpensive for susceptibility testing of isolates of *M. fortuitum* and *M. abscessus* to 7 antimicrobial agents. In comparison with the broth microdilution method approved by CLSI, the colorimetric MIC testing by the modified broth microdilution method using STC could provide a useful means for the antibiotic susceptibility testing for RGM.

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